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FEE TRANSMITTAL for FY 2003 (Small Entity)

Complete if Known	
Application Number	09/439,534
Filing Date	November 12, 1999
First Named Inventor	Simon MOLLER et al.
Examiner Name	Ashwin D. Mehta
Group Art Unit	1638
Attorney Docket Number	2312-103

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METHOD OF PAYMENT (check one)

1. [X] The Commissioner is hereby authorized to charge additional fees and credit any overpayment to Deposit Account Number 02-2135 in the name of Rothwell, Figg, Ernst & Manbeck

   [X] Charge any Additional Fee Required Under 37 CFR 1.16 and 1.17

   [X] Applicant claims small entity status.

2. [X] Payment Enclosed:
   - [X] Check
   - [ ] Credit Card

FEE CALCULATION

1. FILING FEE

Fee Code	Fee $	Fee Description	Fee Paid
2001	375	Utility filing fee	[ ]
2002	165	Design Filing Fee	[ ]
2003	260	Plant Filing Fee	[ ]
2004	375	Reissue Filing Fee	[ ]
2005	80	Provisional Filing Fee	[ ]

SUBTOTAL $

2. CLAIMS

			Extra Claims		Fee	Fee Paid
Total Claims	[ ]	- 20** =	[ ]	x	$ 9 =	[ ]
Independent Claims	[ ]	- 3** =	[ ]	x	42 =	[ ]
Multiple Dependent Claims				+	140 =	[ ]

***or number previously paid, if greater;*

SUBTOTAL $

FEE CALCULATION (continued)

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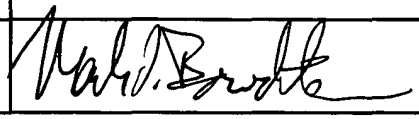
3. ADDITIONAL FEES

Fee Code	Fee Paid	Fee Description	Fee Paid
2051	65	Surcharge - late filing fee or oath	[ ]
2052	25	Surcharge - late provisional filing fee or cover sheet	[ ]
1053	130	Non-English specification	[ ]
1812	2,520	For filing a request for reexamination	[ ]
1804	920	Requesting publication of SIR prior to Examiner action	[ ]
1805	1,840*	Requesting publication of SIR after Examiner action	[ ]
2251	55	Extension for reply within first month	[ ]
2252	205	Extension for reply within second month	[ ]
2253	465	Extension for reply within third month	[465]
2254	725	Extension for reply within fourth month	[ ]
2255	985	Extension for reply within fifth month	[ ]
2401	160	Notice of Appeal	[160]
2402	160	Filing a brief in support of an appeal	[ ]
2403	150	Request for Oral Hearing	[ ]
1451	1,510	Petition to institute a public use proceeding	[ ]
2452	55	Petition to revive -unavoidable	[ ]
2453	650	Petition to revive - unintentional	[ ]
2501	650	Utility issue fee (or reissue)	[ ]
2502	235	Design issue fee	[ ]
2503	315	Plant issue fee	[ ]
1460	130	Petitions to the Commissioner	[ ]
1807	50	Processing fee under 37 CFR 1.17(q)	[ ]
1806	180	Submission of Information Disclosure Statement	[ ]
8021	40	Recording each patent assignment per property (times number of properties)	[ ]
2809	375	Filing a submission after final rejection (37 CFR .129(a))	[ ]
2810	375	For each additional invention to be examined (37 CFR 1.129(b))	[ ]
2801	375	Request for Continued Examination (RCE)	[ ]
1802	900	Request for expedited examination of a design application	[ ]
1504	300	Publication fee for early, voluntary, or normal publication	[ ]
1505	300	Publication fee for republication	[ ]
1455	200	Filing an application for patent term adjustment	[ ]
1456	400	Request for reinstatement of term reduced	[ ]
Other fee (specify)			[ ]

* Reduced by Basic Filing Fee Paid **SUBTOTAL** $625

SUBMITTED BY				Complete (if applicable)	
NAME AND REG. NUMBER	Mark I. Bowditch, Reg. No. 40,315				
SIGNATURE		DATE	July 7, 2003	DEPOSIT ACCOUNT USER ID	

2312-103
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	)	
	)	
Simon MOLLER et al.	)	
	)	
Serial No. 09/439,534	)	Examiner: A. Mehta
	)	
Filed: November 12, 1999	)	Group Art Unit: 1638
	)	
For: INDUCIBLE SITE-SPECIFIC RECOMBINATION FOR THE ACTIVATION AND REMOVAL OF TRANSGENES IN TRANSGENIC PLANTS	)	

AMENDMENT AND REQUEST FOR RECONSIDERATION

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

In response to the Final Office Action mailed January 7, 2003, Applicants submit the following amendments and remarks, and request reconsideration and withdrawal of the pending rejections.

A request for an extension of the time of response by three months accompanies this filing.

AMENDMENTS TO THE CLAIMS are reflected in the listing of claims which begins on page 2 of this paper.

REMARKS begin on page 6 of this paper.

The **CONCLUSION** begins on page 11 of this paper.

AMENDMENTS TO THE CLAIMS

Please cancel claims 40, 42 and 60.

Please amend claims 39, 41, 46 and 59, and add new claims 74 and 75, as indicated.

This listing of claims will replace all prior versions, and listings, of claims in the application.

Listing of Claims:

Claims 1-38 (Canceled)

39. (Currently amended) A plant transformation vector comprising a gene of interest, at least one ~~an~~ inducible gene encoding a recombinase~~, and two~~ under the control of a hormone inducible promoter system, and at least one pair of recombination sites, wherein said recombination sites~~, wherein said recombination sites flank said gene encoding a transcription factor, said marker gene and said inducible gene~~ flank a marker gene.

40. (Canceled)

41. (Currently amended) The vector of claim 39 wherein said pair of recombination sites flanks said marker gene and said inducible gene, and said recombinase causes deletion of said ~~gene encoding a transcription factor, said~~ marker gene and said inducible gene.

42. (Canceled)

43. (Original) The vector of claim 39 wherein said inducible gene encodes CRE, FLP, GIN or R.

44. (Original) The vector of claim 39 wherein said recombination sites are *lox*, *FRT*, *gix* or *RS*.

45. (Original) The vector of claim 44 wherein said *lox* sites are mutant and have a lower affinity for CRE than does wild-type *lox*.

46. (Currently amended) The vector of claim 39 ~~wherein said marker gene is~~ having at least two pairs of recombination sites, wherein a first pair of recombination sites flanks a marker gene under the control of a high affinity hormone inducible promoter, and ~~said inducible~~ a second pair of

recombination sites flanks a recombinase gene ~~is~~ under the control of a low affinity hormone inducible promoter, wherein said high affinity hormone inducible promoter is induced by ~~an~~ a hormone at a low concentration and said low affinity hormone inducible promoter is induced by said ~~inducer~~ hormone at a high concentration.

Claims 47-58 (Canceled)

59. (Currently amended) A method for excising a marker gene from the genome of a ~~transgenic plant or plant cell~~ germ line cell of a transgenic plant, comprising:

a) transfecting a plant ~~or plant~~ cell with the vector of claim 39 to ~~form said transgenic plant or~~ effect stable transformation of said plant cell; ~~and~~

b) ~~adding an inducer to induce said inducible gene, wherein said inducible gene produces a RECOMBINASE which removes said marker gene from said genome.~~ regenerating a transgenic plant from said stably transformed plant cell; and

c) exposing said transgenic plant to an inducer to induce the hormone inducible promoter system,

wherein said inducer induces said hormone inducible promoter

system so as to effect expression of said recombinase in a germ line cell of said transgenic plant, followed by excision of said marker gene from the genome of said germ line cell, such that said marker gene is not inherited by the progeny of said transgenic plant.

60. (Canceled)

Claims 61-71 (Canceled)

72. (Currently amended) A ~~plant or plant cell~~ transformed plant comprising the vector of claim 39.

73. (Canceled)

74. (New) The vector of claim 39, wherein the hormone inducible promoter system is the GVG inducible promoter system.

75. (New) The method of claim 59, wherein the hormone inducible promoter system of the vector is the GVG inducible promoter system.

REMARKS

Claims 39-46, 59, 60 and 72 are pending in the application. All the pending claims were finally rejected in the Office Action mailed January 7, 2003.

I. REJECTION UNDER § 112, FIRST PARAGRAPH, ENABLEMENT

The Patent Office maintained the rejection of claims 39-46, 59, 60 and 72 under 35 U.S.C. § 112, first paragraph, for lack of enablement. This rejection is moot as to cancelled claims 40, 42 and 60. The Patent Office asserts that the specification enables only plant transformation vectors based on the GVG system, and does not provide reasonable enablement for vectors comprising all transcription factors and inducible promoter systems and non-plant vectors.

New claims 74 and 75 specifically recite plant transfection vectors comprising the GVG system, and so are not subject to this rejection. While Applicants maintain that this rejection is not properly applied to the pending claims, in the interest of advancing the prosecution of this application, the claims have been amended to recite plant transformation vectors based on a hormone inducible promoter system. It is believed that the present amendments place the claims in condition for allowance, or in better form for appeal. There is abundant evidence in the

specification showing that hormone inducible promoter systems such as the GVG system function well in the present invention (and the Patent Office acknowledged in the Office Action mailed February 25, 2002, at page 5, that the art teaches that hormone-inducible promoter systems other than GVG would be suitable for use in the invention), and there is no evidence of record that refutes the presumptive accuracy of the disclosure. Subsequent work of the present inventors has in fact confirmed this.[1]

The fact that unrelated inducers, such as heat shock, have subsequently been deemed unsuitable for use does not detract from the enabling nature of the present disclosure, which shows expressly that hormone inducible promoter systems in fact function well in the present invention - it is well within the ordinary skill in the art to select and test appropriate inducible promoters for use in the present vectors and methods, without resort to undue experimentation. See Ex parte Jackson, 217 U.S.P.Q 804, 807 (B.P.A.I. 1982) ("The test [for determining if experimentation is "undue"] is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine ..."); In re Angstadt & Griffin, 190 U.S.P.Q 214,

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[1]See Zuo, et al., Nature Biotech., 19:157-161 (2001), a copy of which is attached for the Examiner's convenience, describing the hormone-inducible "XVE" promoter in similar uses.

218 (C.C.P.A. 1976) (experimentation not undue unless it requires "ingenuity beyond that to be expected of one of ordinary skill in the art."). Furthermore, because operativeness and enablement are separate inquiries,[2] even when some embodiments of an invention are inoperative, the enabling nature of the disclosure as a whole is not negated. Atlas Powder Company v. E.I. Du Pont de Nemours & Company and Alamo Explosives Company, Inc , 750 F.2d 1569, 1576; 224 U.S.P.Q 409, 414 (Fed. Cir. 1984) ("Even if some of the claimed combinations were inoperative, the claims are not necessarily invalid."); Crown Operations Int'l, Ltd., et al. v. Solutia, Inc. 289 F.3d 1367, 1380; 62 U.S.P.Q.2d 1917, ____ (Fed. Cir. 2002) ("Such inoperative embodiments do not necessarily invalidate the claim."). Applicants therefore respectfully request entry of the amendments, and reconsideration and withdrawal of this rejection.

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[2]National Recovery Technologies, Inc. v. Magnetic Separation Systems, Inc., et al., 166 F.3d 1190, 1196; 49 U.S.P.Q.2D 1671, 1676 (Fed. Cir. 1999) ("Whether a patented device or process is operable is a different inquiry than whether a particular claim is enabled by the specification. In order to satisfy the enablement requirement of §§ 112, paragraph 1, the specification must enable one of ordinary skill in the art to practice the claimed invention without undue experimentation."); Crown Operations Int'l, Ltd., et al. v. Solutia, Inc. 289 F.3d 1367, 1380 n. 9; 62 U.S.P.Q.2d 1917, ____ n. 9 (Fed. Cir. 2002) ("The inoperative embodiment inquiry informs the enablement inquiry; they are not the same inquiry.") (citing Nat'l Recovery Technologies, supra.

II. REJECTIONS UNDER 35 U.S.C. § 103(a)

The Patent Office has maintained its rejection of claims 39-44, 59, 60 and 72 under 35 U.S.C. § 103(a) as being unpatentable over Sugita et al. ("Sugita"), in view of Aoyama et al ("Aoyama"). This rejection is moot as to cancelled claims 40, 42 and 60. While Applicants maintain the position that the Patent Office has not made out a case of prima facie obviousness of the claims, in the interest of advancing the prosecution of this application, the claims have been amended in a manner that is believed to define subject matter that is definitively free of the prior art. The claims have now been amended to recite plant transformations vectors employing a hormone inducible promoter system (claims 39, 41, 43-44 and new claim 74), transformed plants comprising such vectors (claim 72) and methods employing such vectors (claims 59 and new claim 75). The prior art does not teach or suggest this particular combination of elements, and a person having ordinary skill in the art would not be motivated to make the modifications to the prior art necessary to arrive at the presently claimed invention with any reasonable expectation of success.

Furthermore, as has been previously stated, the present invention unexpectedly permits reliable, consistent, high-efficiency recombination in plant germ-line cells, something that

prior art methods did not. As previously argued, Sugita et al. were never able to demonstrate excision in germ line cells, and thus were never able to demonstrate that the "excised" phenotype (i.e., the genome lacking the excised gene) was inherited in subsequent generations. See Paper 16 (Amendment file June 25, 2002), pp. 15-16. It is believed that the present amendments place the claims in condition for allowance, or in better form for appeal. New claims 74 and 75 specifically recite plant transfection vectors comprising the GVG system, and so are not subject to this rejection. Applicants therefore respectfully request entry of the amendments, and reconsideration and withdrawal of this rejection.

The Patent Office also maintained its rejection of claim 45 as being obvious over Sugita, in view of Aoyama, and further in view of Albert, et al. For the reasons set forth above with regard to claims 39, 41, 43-44, 59, 72 and new claims 74 and 75, Applicants believe that this claim is now in condition for allowance, and respectfully request that this rejection be reconsidered and withdrawn.

CONCLUSION

In view of the forgoing amendments and remarks, Applicants submit that the claims are in condition for allowance, and request withdrawal of all pending rejections and a favorable action on the claims.

Respectfully submitted,

July 7, 2003 By 

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Chemical-regulated, site-specific DNA excision in transgenic plants

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We have developed a chemical-inducible, site-specific DNA excision system in transgenic *Arabidopsis* plants mediated by the Cre/*loxP* DNA recombination system. Expression of the Cre recombinase was tightly controlled by an estrogen receptor-based fusion transactivator XVE. Upon induction by β-estradiol, sequences encoding the selectable marker, Cre, and XVE sandwiched by two *loxP* sites were excised from the *Arabidopsis* genome, leading to activation of the downstream *GFP* (green fluorescent protein) reporter gene. Genetic and molecular analyses indicated that the system is tightly controlled, showing high-efficiency inducible DNA excision in all 19 transgenic events tested with either single or multiple T-DNA insertions. The system provides a highly reliable method to generate marker-free transgenic plants after transformation through either organogenesis or somatic embryogenesis.

Keywords: DNA excision, Cre/*loxP*, marker-free transformants, transgenic plants, XVE

The use of transgenic technology for agricultural purposes has encountered several challenges. One concern is related to the presence in transgenic crop plants of marker genes conferring antibiotic or herbicide resistance. Although no scientific basis has been determined for these concerns, removal of marker genes would likely hasten the public acceptance of transgenic crops.

To this end, several systems for the generation of marker-free transgenic plants have been described. One rather time-consuming approach is to excise or segregate a marker gene from a target gene by sexual crosses or retransformation[1–9]. A second approach is based on overexpression of the isopentenyl transferase (*ipt*) gene[10,11]. In an early attempt, Ebinuma and coworkers developed an *ipt*-based transformation system with low efficiencies (0.5–1.0%)[12]. Recently, these researchers reported an improved marker-excision system using the R recombinase controlled by an inducible promoter, and obtained five marker-free plants from 37 *ipt*-shooty lines[13]. However, the lack of genetic data makes it uncertain whether DNA recombination had occurred in the germlines of these plants. Moreover, this system is not suitable for most economically important crops, which are regenerated through somatic embryogenesis rather than cytokinin-dependent organogenesis. In an independent effort, Kunkel *et al.*[14] placed *ipt* under the control of the GAL4-VP16-glucocorticoid receptor (GVG)-inducible expression system[15] enabling inducible *ipt* expression and regeneration of transgenic plants. Although this method improved the transformation efficiency, the non-plant 35S-*GVG* and *UAS-ipt* transgenes may also raise public concerns. Additionally, the GVG-*ipt* system can only be used for the limited number of plant species that depend on organogenesis for regeneration.

To develop a reliable system for excision of selectable markers, it is important to distinguish successful DNA recombination events in germline cells from those in somatic cells. Whereas marker excision from somatic cells may be useful for vegetative propagation of transgenic plants[12,13,16], the technology cannot be applied to most crop plants, which are propagated by seeds. To produce marker-free progeny, successful DNA recombination must occur in gametes or their L2 progenitor cells in the shoot apical meristem[17–20]. Therefore, the general utilization of a marker excision system depends largely on the DNA excision efficiency in germline cells.

We present here a chemical-inducible, site-specific DNA excision system in transgenic *Arabidopsis* plants, termed CLX (for Cre/*loxP* DNA excision system), controlled by the XVE system[21]. Compared to previously reported systems, the CLX system is tightly controlled and DNA excision can be induced at high efficiency. More important, this system is useful for all types of explant regeneration either by organogenesis or somatic embryogenesis.

Results

A chemical-inducible Cre/*loxP* DNA recombination system. Our strategy was to transform and regenerate transgenic plants using any conventional selectable marker, and subsequently to remove the marker from the host plant genome by chemically regulated site-specific DNA excision. The XVE-inducible expression system[21] was chosen to construct the CLX system (Fig. 1). The bacteriophage P1 Cre recombinase, which specifically recognizes *loxP* sites both *in vivo*[22] and in plant cells[1,23], was placed under the control of the XVE system. Because the O^{LexA}-46 promoter has background expression in bacterial cells, the Cre coding sequence was interrupted by a short intron to prevent bacterial expression of *cre*. The *cre-int* fusion gene was generated by inserting intron 5 of the *Arabidopsis KOR1* gene[24] between codons 144 and 145 of the *cre* sequence. Nucleotides 4–6 of the intron (TTG) were mutated to AGT to better match the splicing consensus in *Arabidopsis*. A kanamycin-selectable marker was placed between the *XVE* and the *cre* transcription units. These three transcription units were flanked by two *loxP* sites so that β-estradiol-induced DNA recombination would remove all these components, leading to the activation of the downstream *GFP* gene by the G10-90 promoter[25].

The CLX vector pX6-GFP was introduced into *Arabidopsis* by *Agrobacterium*-mediated root culture transformation[26]. Forty-three putative transformed shoots (T$_0$) were generated after four to five weeks of culturing on a kanamycin-containing shoot regeneration medium. Five shoots were transferred onto an inductive medium, and the remaining 38 shoots were transferred onto

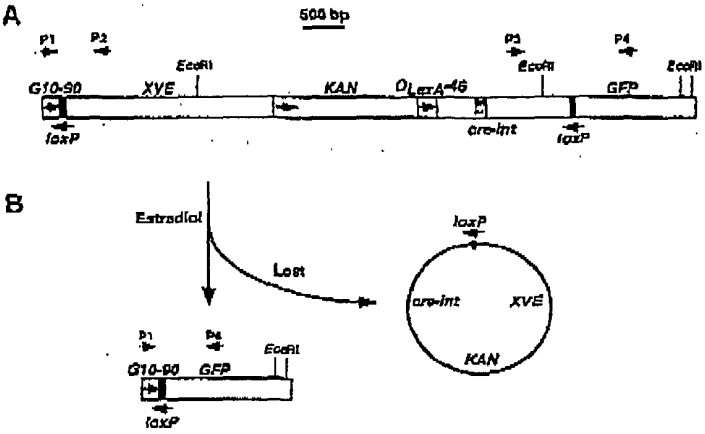

Figure 1. A schematic diagram of the CLX vector and β-estradiol-induced DNA excision. (A) Structural features of the CLX vector pXG-GFP. (See ref. 21 for details of the XVE system.) Three transcription units are located within the two *loxP* sites: *XVE* consists of the coding sequence of the XVE hybrid transactivator terminated by the *rbcs* E9 polyA addition sequence, and is activated by the *G10-90* promoter upstream of the *loxP* site; *KAN* consists of the nopaline synthase (*NOS*) gene promoter, the coding sequence of the neomycin transferase II (NPTII), and the *NOS* polyadenylation sequence; *cre-int* consists of eight copies of the *LexA* operator sequence fused to the −46 CaMV*35S* promoter, the coding sequence of Cre interrupted by an intron and terminated by the *NOS* polyadenylation sequence. Downstream of the second *loxP* site, the *GFP* cDNA was terminated by the *rbcs* 3A polyA addition sequence. Arrows inside squares indicate the direction of transcription. P1 through P4 denote primers used for PCR analysis shown in Figure 2A. Four *Eco*RI sites (used for genomic Southern blot analysis in Fig. 2B) are located at nucleotides 1,796, 5,615, 7,326, and 7,496, respectively. (B) Putative products of the β-estradiol-induced site-specific DNA recombination.

Murashige-Skoog (MS) medium[27]. After induction for two weeks, uniform GFP fluorescence was detected in roots of all five explants cultured on the inductive medium, whereas no *GFP* expression was observed in explants grown on the noninductive MS medium (15 lines examined). All 43 independent lines were then transferred to soil, and T_1 seeds were obtained from 23 lines including 3 lines (numbers 1–3) previously treated with β-estradiol at the T_0 generation. The death before flowering of the remaining 20 lines was presumably due to poor root growth and development.

Genetic analysis of putative T_0 recombinants. T_1 seeds from transgenic lines 1, 2, and 3, treated with β-estradiol in the T_0 generation, were germinated on MS medium and analyzed for *GFP* expression. The proportion of GFP-positive (GFP^+) progeny was less than that expected for a dominant gene (column 2, Table 1). To estimate the recombination frequency, the transgenic locus number was first determined by transferring all GFP-negative (GFP^-) progeny either to the inductive or the selective medium, and additional T_1 progeny showing either β-estradiol-induced *GFP* expression or kanamycin resistance (KAN^R) were identified. It appeared that lines 1 and 3 contained a single transgenic locus, and line 2 contained possibly two transgenic loci (column 3, Table 1). From the number of originally GFP^+ progeny and the transgenic locus numbers, we estimated that DNA recombination had occurred in ~29–66% of germ cells.

In line 2, whereas both transgenic loci appeared to undergo successful DNA recombination upon induction, excision at neither locus was complete. Formation of genetic chimeras in these T_0 transgenic plants could be due to inaccessibility of the L2 progenitor cells to the inducer and inducer instability as well as positional effects of the T-DNA insertions (see below). In lines 1 and 3, T_2 progeny of the putative T_1 recombinants, expressing *GFP* without the inducer, showed a complete loss of the KAN^R marker gene and a mendelian segregation for *GFP* expression in all 17 families tested (Table 1), suggesting complete and precise DNA recombination.

DNA recombination is highly inducible in all tested transgenic lines. To investigate whether the β-estradiol-induced DNA excision occurred in each of the transgenic lines, T_1 seeds from the 20 noninduced transgenic lines (numbers 4–23) were germinated on either the selective or the inductive medium. Four independent lines (numbers 9, 13, 18, and19) appeared to be nontransgenic escapes based on genetic and molecular analyses. No *GFP* expression was detected in the remaining 16 transgenic lines when grown on the selective medium, but all lines showed individuals with uniform *GFP* expression after β-estradiol treatment, indicating that the CLX system in all of these lines was tightly controlled and highly responsive to the inducer. Within each T_1 line, GFP^+ plants were kanamycin-sensitive (KAN^S) after transfer to the selective medium, indicating that they were wild-type progeny. Based on the segregation patterns of both the selection marker and *GFP* expression, four lines (numbers 6, 8, 11, and 22) appeared to contain a single transgenic locus whereas six lines (numbers 4, 5, 7, 12, 14, and 16) apparently contain multiple transgenic loci. In these 10 transgenic lines, both the selection marker and the β-estradiol-induced *GFP* expression segregated in a mendelian manner. The transgene copy number was inherently difficult to determine in lines

Table 1. Genetic analysis of T_1 and T_2 progeny derived from putative T_0 recombinants

	T_1[a]		T_2 (family)[d]		
Line	GFP^+/GFP^- (%)[b]	Segregation of transgene[c]	KAN^R/KAN^S	GFP^+/GFP^- Heterozygous	GFP^+/GFP^- Homozygous
1	18/64 (29.3)	63/19	0/1,274 (8)	941/307 (7)	90/0 (1)
2	33/20 (66.4)	53/0	Variable[e]	Variable[e]	Variable[e]
3	18/26 (54.5)	32/12	0/1,235 (9)	652/211 (6)	240/0 (3)

[a]Grown on MS medium without kanamycin or β-estradiol. *GFP* expression was examined three to seven days after germination.
[b]Excision efficiency (%): the ratio of observed to projected (based on a 3:1 or 15:1 segregation) GFP^+ progeny.
[c]GFP^+ plants were transferred onto a kanamycin-selective or an inductive medium. Additional T_1 progeny that showed KAN^R or *GFP* expression were identified. Therefore, the segregation indicates the ratio of the sum of seedlings showing KAN^R and GFP^+ to those without either trait.
[d]Randomly selected T_1 GFP^+ seedlings were transferred to soil. Seeds were collected from individual T_1 plants and placed on different media. The numbers of tested T_2 families are given in parentheses. KAN^R / KAN^S plants were germinated on the kanamycin-selective medium. KAN^R or KAN^S phenotypes were scored 7–10 days after germination. GFP^+/GFP^+ plants were germinated on MS medium only. Heterozygous or homozygous indicates families showing segregation or no segregation, respectively, for *GFP* expression; therefore, the respective T_1 progenitors were most likely heterozygous or homozygous, respectively, for the transgenic locus. When germinated on the inductive medium, a similar segregation pattern was observed for these families.
[e]Both the kanamycin-selective marker and *GFP* expression showed a variety of segregation patterns including 15:1 (two families), 3:1 (one family), and no segregation (five families).

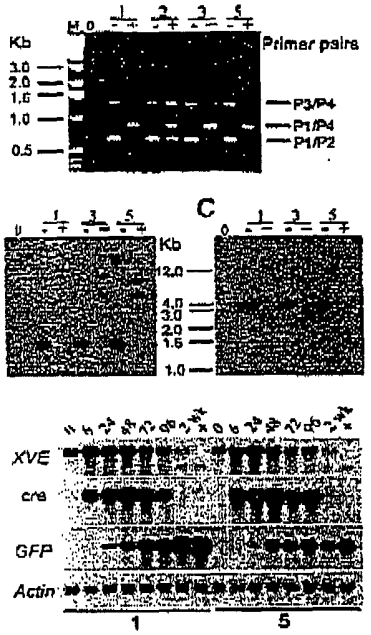

Figure 2. Molecular characterization of β-estradiol-induced site-specific DNA excision in transgenic *Arabidopsis* plants. (A) PCR analysis of genomic DNA prepared from wild-type (lane 0) and transgenic plants (all T_2 plants; lines 1, 2, 3, and 5 as indicated on the top) using primers P1 through P4. The expected PCR products from different combinations of primer pairs are indicated on the left. Uninduced transgenic plants (– lanes) were grown on the selective medium. Putative recombinants (+ lanes) were T_2 progeny (grown on MS medium) derived from β-estradiol-treated T_0 (lines 1, 2, and 3) or T_1 (line 5) transgenic plants. M, DNA molecular weight markers. (B, C) Genomic Southern blot analysis. Blots containing digested genomic DNA (3 μg) from wild-type and transgenic plants were hybridized with either a *GFP* probe (B) or a *NPTII* (C) probe. (D) Northern blot analysis of transgene expression. Two-week-old T_2 plants (lines 1 and 5 as indicated at the bottom) germinated on the selective medium were transferred to the inductive medium and incubated for various time periods (numbered lanes indicate number of hours; 2 wk denotes transgenic plants that were grown on the inductive medium for two weeks; + lanes, same as in part A). Variations in transgene expression in line 5 were presumably caused by the segregation of two transgenic loci among T_2 progeny. Note that the minor band shown in the *XVE* blot is due to residual signal from the *GFP* probe.

10, 15, 17, 20, 21, and 23 because of the small population sizes.

To further characterize the system, we analyzed T_2 progeny from eight transgenic lines (numbers 4, 5, 6, 7, 11, 12, 14, and 16) in detail. The remaining eight lines (numbers 8, 10, 15, 17, 20, 21, 22, and 23), which showed β-estradiol-inducible *GFP* expression in the T_1 generation, were not investigated further. T_2 progeny of the putative T_1 recombinants (treated with β-estradiol and showing *GFP* expression) showed an excision efficiency similar to that of lines 1, 2, and 3. On the other hand, T_1 nonrecombinants showed a characteristic mendelian segregation pattern for both KAN^R and β-estradiol-dependent *GFP* expression (Tables 2 and 3). The above results indicated that the β-estradiol-induced DNA excision occurred in all transgenic lines examined.

Independent DNA recombination in multiple transgenic loci. Segregation analysis suggested that seven transgenic lines (numbers 2, 4, 5, 7, 12, 14, and 16) contained more than one transgenic locus. To address whether all of the transgene copies were excisable upon induction, we selected T_2 families with an approximately 15:1 segregation ratio for KAN^R:KAN^S, which should be heterozygous for both transgenic loci, and tested for the β-estradiol-induced *GFP* expression. If both copies of the transgene could undergo recombination, the *GFP* expression phenotype should also segregate in a 15:1 ratio; or a 3:1 ratio if only one copy underwent recombination. In all seven tested transgenic lines, β-estradiol-induced *GFP* expression showed a segregation pattern similar to that of the KAN^R selective marker (Table 3). Note that the *GFP* reporter gene appeared to uniformly express in the inducer-treated transgenic plants as examined under a fluorescence microscope. Some of these plants, however, were genetic chimeras as shown by subsequent genetic (Table 2 and 3) and molecular analyses (see below). Nevertheless, the above results demonstrated that all T-DNA insertions were fully functional for β-estradiol-induced, independent DNA recombination.

Molecular analyses of β-estradiol-induced DNA recombination. To characterize the β-estradiol-induced DNA recombination at the molecular level, we performed PCR analysis using primers specific for the excised sequences and flanking nonexcised sequences (see Fig. 1). In a nonrecombinant T-DNA, P1/P2 and P3/P4 will amplify two DNA fragments of 653 and 1,376 bp, respectively; P1/P4 will be unable to amplify a 6 kb DNA fragment under the assay conditions. Upon correct DNA recombination, however, P1/P4 will amplify the rejoined *G10-90-lexP-GFP* sequence (990 bp). In uninduced plants, only the P1/P2 and P3/P4 amplified fragments were detected, indicating that the system is tightly controlled. After induction, three DNA fragments were detected in genomic DNA prepared from transgenic line 2 plants containing two transgenic loci. These three amplification products corresponded to the P1/P2 and P3/P4 fragments in nonrecombinant T-DNA and to the P1/P4 fragment in recombinant T-DNA. However, in the remaining three lines tested (single transgenic locus, lines 1 and 3; multiple transgenic loci line 5) only the P1/P4 fragment was detected (Fig. 2A), indicating complete DNA excision. Among other tested lines, three (single transgenic locus lines 6 and 11 and multiple transgenic loci line 14) showed complete DNA excision, and four (transgenic lines 4, 7, 12, and 16; all with multiple transgenic loci) with incomplete DNA excision.

We performed genomic Southern blot analysis to test whether the excised DNA fragment was reinserted elsewhere in the host plant genome. A *GFP* probe detected an *Eco*RI fragment with the expected size in uninduced transgenic plants, whereas larger DNA fragments were detected in recombinant plants (Fig. 2B), presumably representing fusion events between *Arabidopsis* genomic DNA and the remaining *G1090-GFP* transgene (see Fig. 1). Using *XVE*, *cre* (data not shown), and *NPTII* (Fig. 2C) coding sequences as probes, no hybridization signal was detected in recombinant plants, whereas a DNA fragment of the expected size was present in uninduced transgenic plants. This indicates that the excised DNA did not reinsert into the host plant genome. Note that two DNA fragments were detected by the *GFP* probe in line 5, containing two copies of the T-DNA insert, further demonstrating that both copies were indeed functional (Fig. 2B).

Table 2. Genetic analysis of T_2 progeny derived from T_1 nonrecombinants with a single transgenic locus[a]

Line	T_1 Heterozygous (family)		T_1 Homozygous (family)[b]	
	KAN^R/KAN^S	GFP^+/GFP^-	KAN^R/KAN^S	GFP^+/GFP^-
1	203/69 (2)	283/88 (2)	225/0 (2)	161/0 (2)
3	179/57 (2)	278/83 (2)	259/0 (2)	137/0 (2)
6	168/53 (2)	171/56 (2)	179/0 (2)	120/0 (2)
11	250/85 (2)	153/48 (2)	212/0 (2)	120/0 (2)

[a] T_1 plants were grown on the selective medium, and T_2 seeds were used in these experiments. See Table 1 footnotes for other technical details.
[b] No *GFP* expression was detected in any tested KAN^R plants (200 seedlings from eight families).

Table 3. Genetic analysis of T_2 progeny derived from T_1 nonrecombinants with T-DNA insertions in multiple loci[a]

Line	KAN^R/KAN^S (family)	GFP^+/GFP^- (family)
2	248/17 (2)	192/12 (2)
4	90/6 (1)	85/7 (1)
5	256/18 (2)	176/11 (2)
7	113/9 (1)	99/7 (1)
12[b]	115/5 (1)	88/4 (1)
14[b]	123/5 (2)	177/9 (2)
16	263/18 (3)	366/26 (3)

[a]T_1 plants were grown on the selective medium and the resulting T_2 seeds used in these experiments. See Table 1 footnotes for other technical details.
[b]In these two lines, both KAN^R and *GFP* expression appeared to segregate in a ratio greater than 15:1, presumably as a result of linkage between these two transgenic loci.

To monitor the progression of induced DNA recombination, we examined transgene expression by northern blot analysis at different times after β-estradiol treatment (Fig. 2D). The *XVE* fusion gene was expressed in uninduced plants but not in recombinant plants (– lanes). The *XVE* fusion gene, along with the contiguous excisable DNA segment, was lost upon inducer treatment. As expected, *XVE* expression gradually declined upon extended induction. *cre* expression was tightly controlled by the XVE system without detectable expression in uninduced plants, but strong induction after β-estradiol treatment. Similar to that of *XVE*, *cre* expression gradually decreased with time, presumably as a result of transgene excision and/or inducer instability (ref. 21). Presumably, this instability, as well as the partial accessibility of L2 progenitor cells to β-estradiol, lead to the formation of genetic chimeras in some transgenic lines. In contrast to the declining *XVE* and *cre* expression, *GFP* expression, which is strictly dependent on correct DNA recombination, was detectable after 6 h of induction and progressively increased upon prolonged β-estradiol treatment. Consistent with the above, GFP fluorescence was usually detectable after 12–16 h of β-estradiol treatment.

Discussion

Here we describe a tightly regulated and highly efficient site-specific DNA excision system in transgenic *Arabidopsis* plants. This system has several advantages. First, it is tightly controlled by β-estradiol and, moreover, any leaky expression will result in no regeneration of transgenic plants due to the loss of the selectable marker. Second, site-specific DNA excision can be induced at any given time. Third, upon induced DNA excision, all "used" components of the system including the selectable marker and the XVE system itself, will be removed from the host plant genome. This feature is of utmost importance for the generation of marker-free transgenic plants, and for the generation of transgenic plants carrying multiple transgenes. Fourth, upon induced DNA excision, the target gene is permanently activated, a situation that presents key advantages over transiently induced target gene expression (such as the XVE system itself). If desired, the *G10-90* promoter can also be excised by placing a *lox*P site upstream from the promotee, whereas a promoter of interest can be used to control the target gene. Fifth, because the system can use any conventional selectable marker, it is applicable to any transformation method. Finally, because multiple transgene insertions, either linked or independent, occur frequently during plant transformation, the removal of all copies of the selectable marker from the host plant genome is of concern. The CLX system is capable of efficiently excising DNA sequences from multiple T-DNA insertions, whether unlinked or linked.

Compared to the GST-MAT system, in which 14% of the induced-transgenic lines underwent DNA excision[13], the CLX system appeared to function in all 19 transgenic lines examined. More importantly, we provide compelling genetic evidence showing a high efficiency (29–66%). β-estradiol-dependent DNA recombination in germline cells, which was unknown for the GST-MAT system. With appropriate improvements in the induction conditions, a higher DNA excision efficiency may be expected.

Based on the KAN^S and *GFP* expression phenotypes, each of the 19 tested lines responded to the inducer, and showed uniform somatic *GFP* expression. Nevertheless, genetic and molecular analyses revealed that some of the treated lines were genetic chimeras, presumably caused by incomplete DNA excision in the L2 somatic progenitor cells because of inducer inaccessibility or instability[21]. This problem may be obviated by repeated applications of the inducer, particularly to the shoot meristem region. In any case, this disadvantage of the CLX system should not detract from its use for regulated, DNA excision in transgenic crop plants. As demonstrated here with *Arabidopsis* lines 1, 2, and 3, primary transformants can be immediately treated with the inducer to obtain T_1 progeny with all possible segregation patterns, allowing the recovery of transgenic plants with the desired genotype. The appropriate T_1 plants containing only the target gene can be used immediately for outcross with commercial varieties.

Experimental protocol

Plasmid construction. All molecular manipulations were carried out following standard procedures[26]. Pwo DNA polymerase was used in PCR's according to the manufacturer's instructions (Roche Molecular Biochemicals, Indianapolis, IN). To construct pX1, the *Pst*I/*Eco*RI fragment of pMM23 (the Cre coding sequence) and a PCR-generated fragment (the *NOS* poly A addition sequence fused to a *lox*P site; pMM23 primed by CreF/3'-lox2 primers, digested with *Eco*RI) were co-ligated into *Pst*I/*Hin*dII digested pBlueScript vector (Stratagene, La Jolla, CA). A *GFP* cDNA was inserted downstream of the *lox*P site to yield pX-2.

The *cre-int* fusion gene vector pX-3 was constructed by a series of PCR-ligation-PCR reactions. All PCR fragments were treated with T4 DNA kinase before subsequent ligation reactions, which were then directly used for downstream PCR. Appropriate controls with different combinations of PCR primers were included in steps 2 and 3. (1) ligation 1/2: the CreATG/CreQB primed PCR fragment (codons 1–144 of Cre; pX1 as the template) was ligated with a CeInF/CeInB amplified PCR fragment containing intron 5 of the *KOR1* gene[24]. Nucleotides 4–6 of the intron (TTG) were mutated to AGT to better match the splicing consensus in *Arabidopsis*. (2) ligation 3/4: CreATG/CeInB primed ligation 1/2 (Cre 1–144-KOR1 intron 5 fusion fragment) was ligated to CreVF/CreRIB (Val-145 to the end of Cre sequence; pX1 as the template); (3) PCR5: ligation 3/4 was amplified with CreATG/CreRIB primers. The *Eco*RI-digested PCR5 fragment was inserted into *Sma*I/*Eco*RI digested pX2 to yield pX3 consisting of *cre-int-Tnos-lox*P*-CFP* sequences.

The *G10-90-lox*P*-XVE* fusion gene (partial XVE sequence) was made by inserting the LoxT1/ERB1359 primed PCR fragment (treated with T4 DNA kinase; pER8 as the template[21]) into the *Sma*I /*Ecl*136II digested pLsG1090 vector[25] to generate pX4. To construct pX5, the *Mlu*I/*Xho*I fragment of pER10 (containing 3'portion of the *XVE* transcription unit, the kanamycin transcription unit, and the *Olexa1-46* target promoter) and the *Sal*I/*Spe*I fragment of pX3 (containing the *cre-int-Tnos-lox*P*-CFP* sequences) were co-ligated into the *Mlu*I/*Spe*I digested pX4. The CLX vector pX6-CFP was constructed by replacing the *Sse*8387I/*Spe*I fragment of pER8 (ref.21) with that of pX5 (see Fig. 1). Primers used for PCR:

CreF: 5'-CTCGACACACTGCCCCGTCTCGGA-3'
3'-lox2: 5'-GAAGATCTATAAGTTCGTATAATGTATGCTATACGAAGT TATGATCTAGTAACATAGATGACACC-3'
CreATG: 5'-CCCGTCGACATGTCCAATTTACTGACCGTA-3'
CreQB: 5'-CTGGTCGAAATCAGTCGCTTCGAA-3'
CeInF: 5'-GTAAGTCTTCTTTTCCTTTACTCTTATCAG-3'
CeInB: 5'-CTGCCAAAATACAGCAAGCCCGAG-3'
CreVF: 5'-CTTCCTTCACTCATCGAAAATAGCGATC-3'
CreRIB: 5'-GCCTTTCCCCCGATGAATAATATTGATG-3'
LoxT1:5'-TAATAACTTCGTATAGCATACATTATACGAAGTTATGAAT-TAAATCCGGCGGAATGAAA-3'
ERB1359: 5'- GATGAGGAGGAGCTGGGCCAGCCG-3'

The pX6-GFP sequence has been deposited in the GenBank database

(accession number: AF330636).

Plant materials, growth conditions, and plant transformation. The *Wassilewskija* ecotype of *A. thaliana* was used in all experiments. The selective and inductive media contained MS medium[27] plus kanamycin (50 mg/L) or 17-β-estradiol (2 μM), respectively. GFP fluorescence was examined using a Zeiss Axioskop fluorescent microscope.

PCR analysis and genomic Southern blot analysis. Genomic DNA was prepared with the Plant DNAeasy Prep Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Approximately 50 ng of genomic DNA were used for PCR. The reactions were subjected to 94°C for 20 s, 60°C for 30 s, and 72°C for 2 min for 50 cycles. Primers for PCR analyses:

P1: 5'-CCATCTCCACTGACGTAAGGCAT-3'

P2: 5'-CTCGTCAATTCCAAGGGCATCGGT-3'

P3: identical to CreF.

P4: 5'-TTGTATAGTTCATCCATGCCATG-3'

Genomic Southern blot analysis was carried out following standard methods[28]. Briefly, genomic DNA (3 μg) prepared from wild-type and transgenic plants was digested with *Eco*RI, and separated on a 0.8% agarose gel, and blotted onto a nylon membrane (Stratagene). Blots were hybridized with a *GFP* probe and the other with a *NPTII* probe labeled with the Megaprimer DNA Labeling System (Amersham, Piscataway, NJ).

All other methods, including plant growth and transformation, and RNA manipulations, have been described[21,24].

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